Is the Rehydrin *TrDr3* from *Tortula ruralis* Associated with Tolerance to Cold, Salinity, and Reduced pH? Physiological Evaluation of the *TrDr3*-Orthologue, *HdeD* from *Escherichia coli* in Response to Abiotic Stress

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Abstract: **We have employed EST analysis in the resurrection moss *Tortula ruralis* to discover genes that control vegetative desiccation tolerance and describe the characterization of the EST-derived cDNA *TrDr3* (*T*ortula *r*uralis **d**esiccation-stress **r**elated). The deduced polypeptide *TRDR3* has a predicted molecular mass of 25.5 kDa, predicted pI of 6.7, and six transmembrane helical domains. Preliminary expression analyses demonstrate that the *TrDr3* transcript ratio increases in response to slow desiccation relative to the hydrated control in both total and polysomal mRNA (mRNP fraction), which classifies *TrDr3* as a rehydrin. Bioinformatic searches of the electronic databases reveal that *Tortula TRDR3* shares significant similarities to the *hdeD* gene product (**H**NS-**d**ependent **e**xpression) from *Escherichia coli*. The function of the HdeD protein in *E. coli* is unknown, but it is postulated to be involved in a mechanism of acid stress defence. To establish the role of *E. coli HdeD* in abiotic stress tolerance, we determined the log survival percentage from shaking cultures of wild-type bacteria and the isogenic *hdeD* deletion strain (Δ*hdeD*) in the presence of low temperature (28 °C), elevated NaCl (5% (w/v)), or decreased pH (4.5), or all treatments simultaneously. The Δ*hdeD* deletion strain was less sensitive, as compared to wild-type *E. coli,* in response to decreased pH ($p > 0.009$), and the combination of all three stresses ($p > 0.0001$).**

Key words: **Desiccation, membrane protein, moss.**

Introduction

Mosses are a diverse and widely distributed group of land plants that have been particularly successful in colonizing water-limiting environments (Wood, 2005). The resurrection moss *Tortula ruralis* has been studied for more than 30 years as an experimental system for examining the molecular and biochemical mechanisms of vegetative desiccation tolerance in plants (Oliver et al., 2000). Unlike many plant stress responses, the alteration in gene expression within *T. ruralis* gametophores elicited by desiccation stress is primarily regulated at the post-transcriptional level, as a result of differential selection and/or recruitment of mRNAs from a qualitatively constant mRNA pool (Oliver, 1991). Wood and Oliver (1999) demonstrated that many of the transcripts utilized during the rehydration phase following desiccation of gametophores, defined as rehydrins, are sequestered in mRNP particles during desiccation if drying rates are slow. If drying rates are rapid, i.e., to dryness within 1 h, rehydrin transcripts are not sequestered but are synthesized within the first hour following rehydration. Rehydrin transcripts are not, however, novel as they are present at low levels in hydrated gametophytes and, even during rapid desiccation, are somewhat conserved (Oliver, 1991; Scott and Oliver, 1994).

Utilizing desiccated and rehydrated *T. ruralis* gametophores, we have employed EST analysis to discover and analyze a suite of genes associated with vegetative desiccation tolerance (Wood et al., 1999; Wood and Oliver, 2004; Oliver et al., 2004) and our continued efforts have identified a number of cDNAs associated with desiccation stress which we have designated *TrDr* genes (*T*ortula *r*uralis **d**esiccation-stress **r**elated). *TrDr1* and *TrDr2* are significantly similar to *psbI* and the desiccation–stress-related cDNA pcC3-06 from *Craterostigma plantagineum,* respectively (Triwitayakorn and Wood, 2002). *TrDr1* and *TrDr2* steady-state transcript levels increased in response to desiccation and preferentially accumulated within the polysomal mRNA fraction.

A new cDNA clone from *T. ruralis TrDr3* is predicted to encode a novel transmembrane polypeptide that is significantly similar to the HdeD bacterial protein family. In *E. coli* the *H-NS* protein functions as a transcriptional repressor and *hdeD* (***H****NS*-**d**ependent **e**xpression) is one of a suite of genes that exhibit enhanced expression within an *H-NS* deletion background (Yoshida et al., 1993). Bordi et al. (2003) demonstrated that *hdeD* is negatively regulated by the *ToS*/*TorR* phosphorelase system. Subsequent DNA microarray experiments have demonstrated that *hdeD* is an acid-inducible gene, however Δ*hdeD* deletions had no impact upon acid resistance (AR) (Tucker et al., 2002). Interestingly, the *hdeD* deletion abolishes *YdeO*-induced AR (Masuda and Church, 2003). The function of the HdeD protein in *Escherichia coli* is unknown, but it is postulated to be involved in a mechanism of acid stress defence, and expression of the gene is negatively regulated in response to increased pH (Bordi et al., 2003).

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As in many areas of molecular physiology, understanding vegetative desiccation tolerance has been limited by the inability to use genetic analysis of mutants to clearly establish the role of desiccation stress inducible cDNAs in desiccation tolerance. In this report, we describe the bioinformatics analysis of the desiccation stress associated cDNA *TrDr3,* and the physiological characterization of an *E. coli* strain that carries a *TrDr3* orthologue deletion mutant (i.e., the ΔhdeD deletion strain) in response to various abiotic stresses. In this way, we hope to be able to direct our efforts to understanding the role of *TrDr3* in the desiccation tolerance phenotype of *T. ruralis.*

Materials and Methods

Strains, culture conditions, and data analysis

The wild-type *E. coli* strain MG1655 (CGSC 7740) and the isogenic deletion strain ΔhdeD were kindly provided by Dr. T. Conway (University of Oklahoma, Norman OK) (Tucker et al., 2002). Cultures were grown aerobically overnight, with agitation at 240 rpm, in MOPS minimal media (pH 7.4) (Neidhardt et al., 1974). MES minimal media was used for the pH 4.5 media. In all media, glucose (0.2% [w/v]) was the sole carbon source. For the treatments, cultures were inoculated with either 0.5 ml of the overnight culture or 0.5 ml of the diluted overnight culture (1 : 100 in MOPS minimal media), and grown aerobically with agitation at 240 rpm at 37 °C in 50 ml of medium in 125-ml flasks (New Brunswick Scientific Co., model G-76) for 10.5 h. Cultures were continuously sampled at 0 h, 3.5 h, 7.0 h, and 10.5 h by removing either 100 µl or 1000 µl of culture volume without disrupting continuous agitation. 100-µl samples were serially diluted, spread onto LB agar plates, incubated overnight at 37 °C, and the number of colonies was determined. The abiotic stress treatments analyzed were elevated NaCl (5% [w/v]), decreased pH (pH 4.5), or decreased temperature (28 °C). For elevated NaCl, cultures were grown in salinized MOPS minimal media (5% [w/v]) and samples were serially diluted in salinized MOPS minimal media. For decreased temperature, cultures were grown at (28 °C). For decreased pH, cultures were grown in MES minimal media (pH 4.5) and samples were serially diluted in MES minimal media (pH 4.5). For elevated NaCl, decreased temperature and decreased pH, cultures were grown in salinized MES minimal media (5% NaCl [w/v], pH 4.5) at 28 °C, and samples were serially diluted in salinized MES minimal media (5% NaCl [w/v], pH 4.5). *E. coli* growth data were log transformed and presented as the log percentage. Means and standard deviation (n = 4 or n = 3), and Student's two-tailed *t*-test were calculated using Microsoft Office Excel 2003.

Database searches and bioinformatic analyses

PSI-Blast, TBlastN, and TBlastX (low complexity filter on, Blosum62 substitution matrix) were used to search the "nr" "swissprot", and "EST" databases archived at the NCBI (www.ncbi.nlm.nih.gov) (Altschul et al., 1990, 1997). Protein motifs were queried using the CDD (Conserved Domain Database) and CDART (Conserved Domain Architecture Retrieval Tool) tools (Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al., 2005). Iterative sequence similarity searches with the PSI-BLAST program were employed to identify distant homologues (inclusion threshold 0.002). Hsp bit scores greater than 40 were considered significant. Deduced amino acid sequences were aligned using Clustal-X (Thompson et al., 1997) and ESPript (Gouet et al., 1999). The neighbor-joining phlyogram (Saitou and Nei, 1987) was viewed with NJPlot (Perrière and Gouy, 1996) using the following set: *Anabena variabilis* (ZP_001060326), *Bradyrhizobium japonicum* (NP_771030), *E. coli* (NP_756184), *Legionella pneumophila* (YP_096736), *Nostoc punctiforme* (ZP_00106757), *Parachlamydia* sp. (YP_007938), *Physcomitrella patens* (BU052141), *Pseudomonas* sp. (NP_254096), *Ralstonia solanacearum* (CAE27958), *Rhodopseudomonas palustris* (CAE27958).

Results and Discussion

The complete ORF-containing cDNA sequence TrDr3 (AY168202) was derived from the *T. ruralis* EST (AI304984). *TrDr3* is 1010 bp in length, encoding a 233 amino acid-deduced polypeptide (nucleotide 91 – 792), with a predicted molecular mass of 25.5 kDa and pI of 6.7 (Fig. **1 A**). Bioinformatic analysis utilizing BlastX, BlastN, BlastP, and PSI-Blast (Altschul et al., 1990, 1997) demonstrates that *TRDR3* is significantly similar to bacterial sequences from *Rhodopseudomonas palustris, Parachlamydia* sp., *Nostoc punctiforme,* and *Ralstonia solanacearum.* The results of the PSI-blast are presented in Table **1**. The Conserved Domain Database (CDD) tool identifies the deduced polypeptide *TRDR3* as having significant alignment with COG3247 (PSSM-ID 12853, E = 2 e-08) (Table **1**). COG3247 is the HdeD (uncharacterized conserved protein) single domain family comprised of 22 proteins found within 16 prokaryotic species.

We employed iterated PSI-blast in order to delineate those deduced polypeptide sequences significantly related to *TrDr3.* Using TRDR3 as query polypeptide sequence, we identified a cluster of polypeptide sequences comprised solely of bacterially-derived polypeptide sequences-including HdeD from *E. coli* (Table **2**). Using the HdeD homologue from either *N. punctiforme* or *E. coli* as query polypeptide sequence, identified a nearly identical cluster of bacterially-derived polypeptide sequences, with *TrDr3* being the sole eukaryotic sequence (Table **2**). In all three searches, no further significant "hits" were obtained after either 5 or 6 iterations. Finally, BlastN search of the "EST" database identifies a series of significant "hits" only with moss-derived cDNA sequences – 14 Tor sequences from *T. ruralis* (Oliver et al., 2004) and 2 cDNA sequences from *Physcomitrella patens* (Machuka et al., 1999) (Table **3**). No other eukaryotic nucleotide sequences were demonstrated to have significant similarity to either *TrDr3* or other bacterial COG3247 members. In overlapping regions that range from 527 – 720 nucleotides, *TrDr3* is 97 – 99% identical to EST sequences from *Tortula* (E value = 0.0 for each sequence). When the *TrDr3* nucleotide sequence is aligned with the closest related bacterial hdeD nucleotide sequence (on a deduced polypeptide sequence basis), the level of identity is only 45.2%. Based upon these data, *TrDr3* is a plant-derived cDNA and not a bacterial gene that has contaminated the library.

The *TrDr3* nucleotide sequence corresponds to three independent EST clusters within the *Tortula* rehydration EST collection, comprised of 9159 high quality ESTs that form 5.563 EST clusters, as described by Oliver et al., 2004. Cluster 158 is comprised of Tor9178, Tor9900, Tor6277, Tor9156, Tor9388, Tor3964, Tor9835, Tor6678. Cluster 1021 is comprised of Tor9003, Tor2940, and Cluster 1022 is comprised of Tor10234,

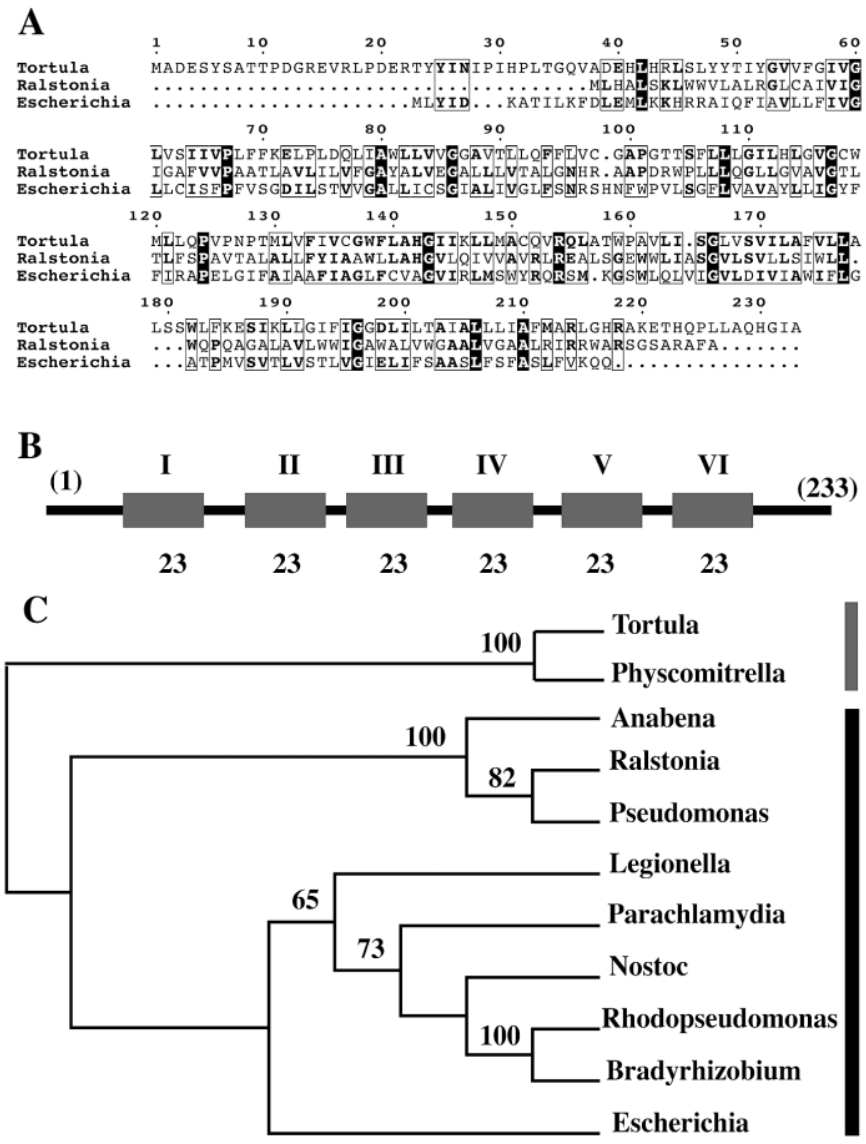

Fig. 1 (**A**) Alignment of the deduced polypeptide sequence of *Tortula ruralis TrDr3* (AY168202) with orthologues from *Ralstonia solanacearum* (NP_521987) and *Escherichia coli* (*HdeD,* NP_756184). (**B**) Schematic depicting the predicted transmembrane helices within *TrDr3*. (**C**) Phylogram derived from a data set of deduced polypeptides for *TrDr3,* HdeD, and 10 related deduced polypeptides. Sequences are numbered from the presumed translation initiation methionine (M) and are aligned using ClustalX (Thompson et al., 1997) to give maximal alignment; open boxes and shaded boxes indicate conserved and identical amino acid residues, respectively (**A**). Solid grey bars indicate a transmembrane helix (**B**). The tree was constructed using Clustal-X. See "Materials and Methods" for the data set (**C**). Numbers above the lines represent bootstrap percentages (based on 1000 replicates). The black bar indicates bacterial species, and the grey bar indicates moss species.

Tor8527, Tor3480, and Tor10459 (Table **3**). These three clusters do not assemble into a single sequence even though they appear to represent transcripts from an individual gene. This could simply be the result of non-overlapping sequences representing different parts of the *TrDr3* gene or it is possible that *TrDr3* is a member of a small gene family with significant sequence diversity to prevent assembly into a single cluster.

Alignment of the *TRDR3*-deduced polypeptide sequence with related bacterial polypeptide sequences gives 15 conserved amino acid residues and several blocks of conserved residues (Fig. **1 A**). The deduced polypeptide is predicted by TMHMM (Krogh et al., 2001) to contain six transmembrane helical domains (residues 48 – 70, 76 – 98, 104 – 126, 131 – 153, 161 – 183, and 191 – 213), each 23 amino acid residues in length, with no predicted transit sequence(s) (Fig. **1 B**). To examine the structural relationship between *TRDR3* and related bacterial sequences, the deduced amino acid sequences were analyzed by the neighbor-joining method (Saitou and Nei, 1987). The gene tree assembled from the pairwise alignment of those sequences is depicted in Fig. **1 C**. The two moss sequences reliably grouped as a single clade with 100% bootstrap support, while the bacterial sequences form two distinct clades. Although a novel deduced polypeptide sequence, *TRDR3,* is clearly related to these bacterial sequences. Our results indicate that *TrDr3*-related sequences are confined to bacteria and mosses. We hypothesize that *TrDr3* represents a cyanobacterial-derived plant gene that has been lost in tracheophytes. To our knowledge, *TrDr3* is one of the first examples of a "moss-retained" gene sequence and may provide greater insight into the evolution of both moss and angiosperm genomes.

As part of an ongoing analysis of gene expression in *Tortula ruralis* associated with the response of this bryophyte to desiccation and rehydration, we have constructed a *Tortula* rehydration cDNA array that has been utilized in an expression profiling study (M. J. Oliver, unpublished data). The *Tortula* rehydration cDNA array contains sequences that represent all 5563

Table 1 PSI-Blast search of the "nr" and "swissprot" databases using *T. ruralis TrDr3* deduced polypeptide (AAN8738) as query sequence

Species	Accession number	Gene name	COG	Score
Rhodopseudomonas palustris	NP_947859	conserved protein	3247 *(HdeD)*	46 (6e-04)
Parachlamydia sp.	YP_007938	Pc0939	3247 *(HdeD)*	46 (7e-04)
Nostoc punctiforme	ZP_00106757	conserved protein	3247 *(HdeD)*	44 (0.002)
Ralstonia solanacearum	NP_521987	hypothetical protein	3247 *(HdeD)*	43 (0.004)
Escherichia coli	NP_756184	*hdeD*	3247 *(HdeD)*	ns

ns = not significant

Table 2 Iterated PSI-Blast searches of the "nr" and "swissprot" databases using *T. ruralis TrDr3*, *N. punctiforme* (conserved protein), or *E. coli (HdeD)* deduced polypeptides as query sequence

Species	Accession number	COG	*T. ruralis (TrDr3)* 5 iterations Score	*N. punctiforme* 5 iterations Score	*E. coli (HdeD)* 6 iterations Score
R. palustris	NP_947859	3247 *(HdeD)*	132 (5e-30)	106 (3e-22)	101 (1e-20)
Parachlamydia sp.	YP_007938	3247 *(HdeD)*	120 (2e-36)	105 (7e-22)	97 (2e-19)
N. punctiforme	ZP_00106757	3247 *(HdeD)*	152 (5e-36)	144 (9e-34)	105 (5e-22)
R. solanacearum	NP_521987	3247 *(HdeD)*	93 (5e-18)	78 (1e-14)	61 (2e-8)
E. coli	NP_756184	3247 *(HdeD)*	60 (3e-8)	74 (1e-12)	150 (3e-35)
T. ruralis	AAN87348	na	253 (2e-66)	87 (2e-16)	56 (6e-7)

na = not applicable

Table 3 BlastN search of the "EST" databases using *T. ruralis TrDr3* cDNA as query sequence

Species	Accession number	cDNA name	Tortula cluster [a]	Score [b]	Overlap length and % identity
T. ruralis	CN208506	Tor9003	1021	1314 (0.0)	690/699 (98%)
	CN208684	Tor2940	1021	1253 (0.0)	679/690 (98%)
	CN209388	Tor9900	158	1239 (0.0)	664/673 (98%)
	CN205863	Tor6277	158	1324 (0.0)	720/732 (98%)
	CN202750	Tor9178	158	1316 (0.0)	694/704 (98%)
	CN208661	Tor9156	158	1140 (0.0)	599/607 (98%)
	CN208883	Tor9388	158	1154 (0.0)	637/654 (97%)
	CN203666	Tor3964	158	1310 (0.0)	689/697 (98%)
	CN206246	Tor9835	158	1090 (0.0)	615/633 (97%)
	CN200788	Tor6678	158	1039 (0.0)	527/528 (99%)
	CN200564	Tor10234	1022	1195 (0.0)	641/651 (98%)
	CN208047	Tor8527	1022	1209 (0.0)	658/674 (97%)
	CN203233	Tor3480	1022	1277 (0.0)	684/696 (98%)
	CN209318	Tor10459	1022	1033 (0.0)	531/533 (99%)
P. patens	BU052141	PPAS020719	na	121 (2e-24)	277/349 (79%)
	AJ225518	clone 7708	na	107 (4e-20)	93/106 (87%)

[a] Oliver et al., 2004; [b] Hsp bit score and E value; na = not applicable

clusters, along with previously isolated cDNAs (Wood et al., 1999) as expression profile controls. Each of the three *TrDr3* clusters responds to desiccation and rehydration of *Tortula* gametophores in an identical fashion, perhaps indicating that the clusters represent a single *TrDr3* gene. Transcripts for each cluster accumulate in gametophytes during slow drying, when compared to transcript levels present in the hydrated controls. The signal ratio for the treatment (slow-dried total or polysomal RNA) compared to the control (hydrated total or polysomal RNA) is significantly different from and greater than 1.0 for each cluster; approximately 2.7 for the total RNA comparison and 1.75 for the polysomal comparison (M. J. Oliver, unpublished results). This indicates that the *TrDr3* transcripts not only accumulate during slow drying but are also sequestered in the polysomal fraction, previously demonstrated to consist of mRNP particles (Wood and Oliver, 1999), to a greater extent than in hydrated controls. The ratio of signal for the treatment versus control for the samples derived from rapidly

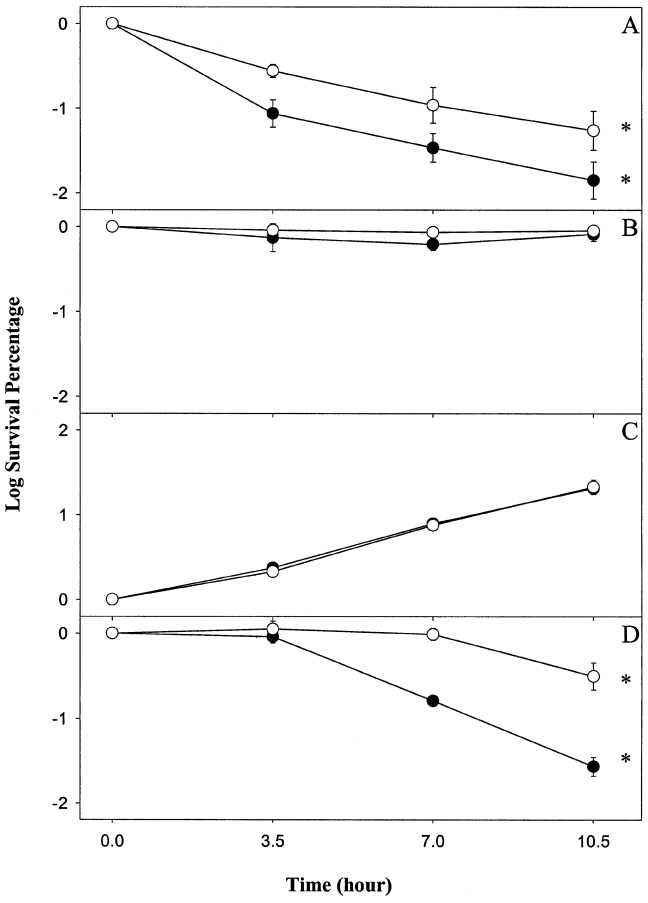

Fig. 2 Relative survival of abiotic-stress-challenged bacteria. (**A**) Acidic growth conditions – pH 4.5. (**B**) Elevated NaCl (5% [w/v]). (**C**) Decreased temperature. (**D**) Decreased temperature, acidic growth conditions, and elevated salinity – 28 °C, pH 4.5, and 5% NaCl (w/v). Closed circles represent wild-type *E. coli* strain MG1655 and open circles represent the isogenic deletion strain Δ*hdeD*. Error bars represent standard deviation (STD); for **A, C, D** n = 4, for **B** n = 3. Asterisks (∗) in **A, D** at 10.5 h indicate statistically significant differences as determined by the Student's *t*-test (**A** $p > 0.009$, **B** $p > 0.0001$).

dried and rehydrated (for 2 h) gametophytes do not significantly differ from 1.0, indicating that at 2 h following rehydration of rapid drying, transcript levels are at control levels (M. J. Oliver, unpublished results).

To establish the role of *E. coli hdeD* in abiotic stress tolerance within bacteria, we determined the log survival percentage from shaking cultures of the wild-type *E. coli* strain MG1655 and the isogenic deletion strain Δ*hdeD* (Tucker et al., 2002) under acidic growth conditions (Fig. **2A**), elevated NaCl (Fig. **2B**), decreased temperature (Fig. **2C**), or under acidic growth conditions, elevated NaCl, and decreased temperature (Fig. **2D**). Under acidic growth conditions, the log survival percentage declined in both cultures (0 – 10.5 h), however, the Δ*hdeD* strain was significantly less sensitive to acid stress ($p > 0.009$ at 10.5 h) (Fig. **2A**). The wild-type *E. coli* and Δ*hdeD* deletion strain behaved similarly in response to elevated NaCl (Fig. **2B**) and decreased temperature (Fig. **2C**). In response to all three stresses applied simultaneously (i.e., pH 4.5, 5% NaCl [w/v] and 28 °C), the log survival percentage declined in both cultures (0 – 10.5 h), and the Δ*hdeD* deletion strain was significantly less sensitive as compared to wild-type *E. coli* ($p > 0.0001$ at 10.5 h) (Fig. **2D**).

The role of the *hdeD* gene in the acid response (AR) is unclear. Previous AR experiments demonstrating that the Δ*hdeD* deletion had no impact upon AR were limited to 2 h of stress treatment (Tucker et al., 2002). In contrast, we have demonstrated a modest but significant increase in AR with the Δ*hdeD* deletion strain up to 10.5 h (Fig. **2A**). Interestingly, AR of the Δ*hdeD* deletion strain was enhanced by incubation in elevated NaCl and at decreased temperature (Fig. **2D**). HdeD is predicted to be a 6 transmembrane protein localized to the plasma membrane; however, HdeD and all members of COG3247, including *TrDr3,* have no known enzymatic activity. Based on these data, we do not think that *hdeD* encodes a membrane transporter. How can the loss of an acid-induced gene lead to enhanced survival under acid growth conditions? The most parsimonious explanation is that HdeD is a metabolically expensive protein to generate, and that the Δ*hdeD* deletion strain is more fit under abiotic stress because it does not carry the extra burden of producing and inserting the *hde* protein.

Conclusion

We have speculated that poikilohydric desiccation-tolerant plants, such as *T. ruralis,* are most similar to the highly tolerant early land plants and offer novel genes potentially lost during angiosperm evolution (Wood, 2005). Our data from *HdeD* suggest that *TrDr3* (as an *HdeD* orthologue) may play a role in cellular salinity, cold, and acid stress tolerance. The expression characteristics of *TrDr3* and the fact that it is represented by a relatively large number of ESTs in the non-normalized *Tortula* rehydration library, as described by Oliver et al., 2004, suggests that it may play a role at the cellular level in dehydration tolerance in mosses. Future experiments will further characterize *TrDr3* expression, pursue the idea that *TrDr3* is an endosymbiont-derived, moss-retained plant gene, and investigate the adaptive role of this novel membrane protein in salinity, cold, and desiccation tolerance of mosses.

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